1: Circ Res. 2003 Apr 4;92(6):579-82. Related Articles, Links

Comment on:

- Circ Res. 2003 Apr 4;92(6):692-9.

Primitive cells and tissue regeneration.

Anversa P, Kajstura J, Nadal-Ginard B, Leri A.

Publication Types:

- Comment
- Editorial

PMID: 12676808 [PubMed - indexed for MEDLINE]

1: J Pathol. 2002 Jul;197(4):468-78. Related Articles, Links

Cardiac stem cells.

Hughes S.

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Augmentation of myocardial performance in experimental models of therapeutic infarction and heart failure has been achieved by the transplantation of exogenous cells into damaged myocardium, a procedure known as cellular cardiomyoplasty (CCM). Historically, a wide range of cell types have been used for CCM, including rat and human fetal ventricular myocytes, but the availability of human fetal donor cells for clinical purposes is limited. The quest for suitable alternative donor cells has prompted research into the use of both embryonic stem (ES) cells and adult somatic stem cells, but the optimal choice of donor cell source is not yet known. Recently, there has been a growing body of evidence that multipotent somatic stem cells in adult bone marrow exhibit tremendous functional plasticity and can reprogramme in a new environmental tissue niche to give rise to cell lineages specific for the new organ site. This phenomenon has made a huge impact on myocardial biology and has captured the imagination of scientists who have recently discovered that multipotent adult bone marrow haematopoeitic stem cells and mesenchymal stem cells can repopulate infarcted rodent myocardium and differentiate into both cardiomyocytes and new blood vessels. These data, coupled with the identification of a putative primitive cardiac stem cell population in the adult human heart, may pave the way for novel therapeutic modalities for enhancing myocardial performance and treating end-stage cardiac disease.

Publication Types:
- Review
- Review, Tutorial

PMID: 12115863 [PubMed - indexed for MEDLINE]

See application 09/919,979 for reference

1: Blood. 1993 Aug 1;82(3):762-70. Related Articles, Links

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Purification and characterization of heterogeneous pluripotent hematopoietic stem cell populations expressing high levels of c-kit receptor.

Orlic D, Fischer R, Nishikawa S, Nienhuis AW, Bodine DM.

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Mouse pluripotent hematopoietic stem cells (PHSC) were fractionated based on size and density using counterflow centrifugal elutriation (CCE). These heterogeneous PHSC populations were further enriched by subtraction of cells with lineage-specific markers (Lin-) followed by positive sorting for c-kit expression. The cells were characterized for their functional and biochemical properties. We defined a subpopulation of c-kit-positive cells that expressed high numbers of c-kit receptors (c-kitBR). One hundred c-kitBR cells from either low- or higher-density fractions were sufficient to repopulate the lymphohematopoietic system in WBB6F1-W/Wv (W/Wv) recipients, whereas no PHSC were found in cells with low (c-kitDULL) or no (c-kitNEG) c-kit expression. Lin- c-kitBR cells were separated into RhoDULL and RhoBR subsets based on their ability to efflux rhodamine 123 (Rho). The PHSC were concentrated in Lin- c-kitBR RhoDULL cells and the number of Lin- c-kitBR RhoBR cells correlated directly with the number of day 12 colony-forming unit-spleen (CFU-S12) in each fraction. We were not able to enrich further for PHSC using monoclonal antibodies to the cell-surface markers AA4.1 or CD4, which have been used by others to isolate PHSC. The small, low-density Lin- c-kitBR subset contained PHSC and few CFU-S12. This enabled us to assay PHSC for expression of the flk-2 gene, which encodes a tyrosine kinase receptor present on fetal liver PHSC. Purified RNA from the low-density Lin- c-kitBR subset did not contain flk-2 mRNA. We suggest that AA4.1, CD4 and flk-2 are expressed as stage-specific markers on PHSC in cell cycle.

PMID: 7687891 [PubMed - indexed for MEDLINE]

1: Cytotherapy. 2002;4(6):521-5. Related Articles, Links

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Adult BM stem cells regenerate mouse myocardium.

Orlic D.

Genetics and Molecular Biology Branch, National Human Genome Research Institute, NIH, Bethesda, MD, USA.

PMID: 12568987 [PubMed - in process]

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Related Resources

1: Pediatr Transplant. 2003 Apr;7(3 Suppl):86-8. Related Articles, Links

Bone marrow stem cells regenerate infarcted myocardium.

Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P.

Genetics and Molecular Biology Branch, NHGRI, NIH, Bethesda, MD 20892, Department of Medicine, New York Medical College, Valhalla, NY 10595, USA.

Heart disease is the leading cause of death in the United States for both men and women. Nearly 50% of all cardiovascular deaths result from coronary artery disease. Occlusion of the left coronary artery leads to ischemia, infarction, necrosis of the affected myocardial tissue followed by scar formation and loss of function. Although myocytes in the surviving myocardium undergo hypertrophy and cell division occurs in the border area of the dead tissue, myocardial infarcts do not regenerate and eventually result in the death of the individual. Numerous attempts have been made to repair damaged myocardium in animal models and in humans. Bone marrow stem cells (BMSC) retain the ability throughout adult life to self-renew and differentiate into cells of all blood lineages. These adult BMSC have recently been shown to have the capacity to differentiate into multiple specific cell types in tissues other than bone marrow. Our research is focused on the capacity of BMSC to form new cardiac myocytes and coronary vessels following an induced myocardial infarct in adult mice. In this paper we will review the data we have previously published from studies on the regenerative capacity of BMSC in acute ischemic myocardial injury. In one experiment donor BMSC were injected directly into the healthy myocardium adjacent to the injured area of the left ventricle. In the second experiment, mice were treated with cytokines to mobilize their BMSC into the circulation on the theory that the stem cells would traffic to the myocardial infarct. In both experimental protocols, the BMSC gave rise to new cardiac myocytes and coronary blood vessels. This BMSC-derived myocardial regeneration resulted in improved cardiac function and survival.

PMID: 12603699 [PubMed - in process]

Current Opinion in Pediatrics 2002; 14(5):627-633

New frontiers in molecular pediatric cardiology

Ellen Dees, MD; H. Scott Baldwin, MD

Numerous advances in understanding the molecular basis of congenital heart disease have been published in the past year. Highlights are reviewed, focusing on two major topics: genetic syndromes and cardiac organogenesis. Genetic syndromes are discussed in the context of complementary data from targeted mutations in animals and genetic mapping studies in humans. These include the DiGeorge, Holt-Oram, Alagille, familial primary pulmonary hypertension, and Noonan syndromes. Novel concepts in cardiac organogenesis are discussed, including the existence and contribution of an anterior heart field to the developing cardiac outflow tract, novel cell-cell signaling involving migrating neural crest, the origins of the conduction system and initial embryonic heartbeat, and the possibility of a population of cardiac stem cells in the adult heart. The studies reviewed have potential clinical relevance in the near future and will be of interest to the clinician interested in congenital heart disease.

Abbreviations

BMPR2 bone morphogenic protein receptor type 2

PPH primary pulmonary hypertension

Sema3C semaphorin 3C

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Current Opinion in Pediatrics 2002; 14(5):627-633

1: Int J Hematol. 2002 Aug;76 Suppl 1:144-5. Related Articles, Links

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Stem cell repair in ischemic heart disease: an experimental model.

Orlic D.

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Bone marrow stem cells (BMSC) from adult mice are now believed to generate non-hematopoietic cell types. This newly defined property is referred to as stem cell plasticity. We tested the potential of lineage negative c-kit positive (Lin- c-kit+), GFP+ BMSC to differentiate into cardiac myocytes in myocardial infarcts produced by ligation of the left coronary artery. At 9 days post-transplant the hearts showed a band of developing GFP+ myocytes within the damaged myocardium. These GFP+ myocytes were positive for cardiac specific myosin and early expressed transcription factors. Endothelial cells and smooth muscle cells also developed from the donor bone marrow cells. Left ventricular end diastolic pressure (LVEDP) and left ventricular developed pressure (LVDP) were improved. Lin-c- kit- cells did not regenerate myocardium. We next tested the ability of cytokine-mobilized BMSC to regenerate myocardium. Nuclei in regenerating cardiomyocytes were positive for Csx/Nkx 2.5, GATA-4 and MEF2. Cytoplasmic proteins included desmin, nestin and connexin 43. Regenerating arterioles consisted of endothelial cells and smooth muscle cells positive for Ki67, and flkl. These regenerating vessels contained circulating TER119 positive red blood cells. Repair of infarcted myocardium resulted in improved heart function and survival. At day 27 after cytokine treatment and surgery, 11 of 15 mice survived compared with 9 of 52 non-treated mice. Left ventricular ejection fraction in infarcted hearts in cytokine-treated mice was 48%, 62% and 114% higher than the ejection fraction in non-treated mice at 9, 16 and 26 days following coronary artery occlusion. These findings demonstrate that circulating autologous stem cells traffic to the ischemic, infarcted myocardium and undergo differentiation into cardiomyocytes and vascular structures. We conclude that adult BMSC have the potential for repair in acute, ischemic heart disease.

PMID: 12430844 [PubMed - in process]

1: Blood. 1994 Sep 1;84(5):1482-91. Related Articles, Links

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Efficient retrovirus transduction of mouse pluripotent hematopoietic stem cells mobilized into the peripheral blood by treatment with granulocyte colony-stimulating factor and stem cell factor.

Bodine DM, Seidel NE, Gale MS, Nienhuis AW, Orlic D.

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Cytokine-mobilized peripheral blood cells have been shown to participate in hematopoietic recovery after bone marrow (BM) transplantation, and are proposed to be useful targets for retrovirus-mediated gene transfer protocols. We treated mice with granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) to mobilize hematopoietic progenitor cells into the peripheral blood. These cells were analyzed for the number and frequency of pluripotent hematopoietic stem cells (PHSC). We found that splenectomized animals treated for 5 days with G-CSF and SCF showed a threefold increase in the absolute number of PHSC over normal mice. The number of peripheral-blood PHSC increased 250-fold from 29 per untreated mouse to 7,200 in peripheral-blood PHSC in splenectomized animals treated for 5 days with G-CSF and SCF. Peripheral blood PHSC mobilized by treatment with G-CSF and SCF were analyzed for their ability to be transduced by retroviral vectors. Peripheral-blood PHSC from splenectomized animals G-CSF and SCF were transduced with a recombinant retrovirus containing the human MDR-1 gene. The frequency of gene transfer into peripheral blood PHSC from animals treated for 5 and 7 days was two-fold and threefold higher than gene transfer into PHSC from the BM of 5-fluorouracil-treated mice ($P < .01$). We conclude that peripheral blood stem cells mobilized by treatment with G-CSF and SCF are excellent targets for retrovirus-mediated gene transfer.

PMID: 7520774 [PubMed - indexed for MEDLINE]

Stem cells for myocardial regeneration.

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Stem cells are being investigated for their potential use in regenerative medicine. A series of remarkable studies suggested that adult stem cells undergo novel patterns of development by a process referred to as transdifferentiation or plasticity. These observations fueled an exciting period of discovery and high expectations followed by controversy that emerged from data suggesting cell-cell fusion as an alternate interpretation for transdifferentiation. However, data supporting stem cell plasticity are extensive and cannot be easily dismissed. Myocardial regeneration is perhaps the most widely studied and debated example of stem cell plasticity. Early reports from animal and clinical investigations disagree on the extent of myocardial renewal in adults, but evidence indicates that cardiomyocytes are generated in what was previously considered a postmitotic organ. On the basis of postmortem microscopic analysis, it is proposed that renewal is achieved by stem cells that infiltrate normal and infarcted myocardium. To further understand the role of stem cells in regeneration, it is incumbent on us to develop instrumentation and technologies to monitor myocardial repair over time in large animal models. This may be achieved by tracking labeled stem cells as they migrate into myocardial infarctions. In addition, we must begin to identify the environmental cues that are needed for stem cell trafficking and we must define the genetic and cellular mechanisms that initiate transdifferentiation. Only then will we be able to regulate this process and begin to realize the full potential of stem cells in regenerative medicine.

Publication Types:
- Review
- Review, Tutorial

Mobilized bone marrow cells repair the infarcted heart, improving function and survival

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Edited by Eugene Braunwald, Partners HealthCare System, Inc., Boston, MA, and approved June 29, 2001 (received for review April 11, 2001)

Attempts to repair myocardial infarcts by transplanting cardiomyocytes or skeletal myoblasts have failed to reconstitute healthy myocardium and coronary vessels integrated structurally and functionally with the remaining viable portion of the ventricular wall. The recently discovered growth and transdifferentiation potential of primitive bone marrow cells (BMC) prompted us, in an earlier study, to inject in the border zone of acute infarcts Lin$^-$ c-*kit*^{POS} BMC from syngeneic animals. These BMC differentiated into myocytes and vascular structures, ameliorating the function of the infarcted heart. Two critical determinants seem to be required for the transdifferentiation of primitive BMC: tissue damage and a high level of pluripotent cells. On this basis, we hypothesized here that BMC, mobilized by stem cell factor and granulocyte-colony stimulating factor, would home to the infarcted region, replicate, differentiate, and ultimately promote myocardial repair. We report that, in the presence of an acute myocardial infarct, cytokine-mediated translocation of BMC resulted in a significant degree of tissue regeneration 27 days later. Cytokine-induced cardiac repair decreased mortality by 68%, infarct size by 40%, cavitary dilation by 26%, and diastolic stress by 70%. Ejection fraction progressively increased and hemodynamics significantly improved as a consequence of the formation of 15 × 10^6 new myocytes with arterioles and capillaries connected with the circulation of the unaffected ventricle. In conclusion, mobilization of primitive BMC by cytokines might offer a noninvasive therapeutic strategy for the regeneration of the myocardium lost as a result of ischemic heart disease and, perhaps, other forms of cardiac pathology.

Sudden occlusion of a major coronary artery and acute myocardial ischemia lead to rapid death of myocytes (M) and vascular structures in the supplied region of the ventricle. Despite the demonstration that a subpopulation of cardiac muscle cells is able to replicate (1), and new vessels are formed (2), this regeneration is restricted to the viable myocardium. The loss of M, arterioles, and capillaries in the infarcted area is irreversible, resulting with time in the formation of scarred tissue. For this reason, most experimental and clinical therapies have mainly focused on limiting infarct size. Attempts to replace the necrotic zone of the heart by transplanting cardiomyocytes or skeletal myoblasts (3–7), although successful in the survival of many of the grafted cells, have invariably failed to reconstitute healthy myocardium and coronary vessels integrated structurally and functionally with the spared ventricular wall.

The recognition that stem cells, particularly those from the bone marrow, have the capacity to colonize different tissues, proliferate, and transdifferentiate into cell lineages of the host organ (8, 9), prompted us in an earlier study (10) to inject Lin$^-$ c-*kit*^{POS} bone marrow cells (BMC) in the contracting myocardium bordering acute infarcts. Surprisingly, the implanted BMC differentiated into M and coronary vessels ameliorating the function of the injured heart (10). This approach, however, required a surgical intervention that was accompanied by high mortality and a grafting success rate of 40%. Therefore, the identification and utilization of a noninvasive method would be highly desirable. Two main determinants seem to be critical for colonization and transdifferentiation of BMC into a variety of tissues: recent damage and a high number of circulating stem cells (8, 9, 11, 12). On this basis, we hypothesized that a sufficient number of BMC mobilized by stem cell factor (SCF) and granulocyte-colony-stimulating factor (G-CSF; refs. 13 and 14) would home to the infarcted heart and promote cardiac repair. To test this possibility, mice were injected with SCF and G-CSF to increase the number of circulating stem cells from 29 in nontreated controls to 7,200 in cytokine-treated mice (13).

Materials and Methods

Myocardial Infarction (MI) and Cytokines. C57BL/6 male mice at 2 months of age were splenectomized and 2 weeks later were injected s.c. with recombinant rat SCF, 200 μg/kg/day, and recombinant human G-CSF, 50 μg/kg/day (Amgen Biologicals), once a day for 5 days (13, 14). Under ether anesthesia, the left ventricle (LV) was exposed and the coronary artery was ligated (10, 15, 16). SCF and G-CSF were given for 3 more days. Controls consisted of splenectomized infarcted and sham-operated (SO) mice injected with saline. BrdUrd, 50 mg/kg body weight, was given once a day for 13 days before the mice were killed; mice were killed at 27 days. Protocols were approved by New York Medical College.

Echocardiography and Hemodynamics. Echocardiography was performed in conscious mice by using a Sequoia 256c (Acuson, Mountain View, CA) equipped with a 13-MHz linear transducer (15L8). The anterior chest area was shaved and two- dimensional (2D) images and M-mode tracings were recorded from the parasternal short axis view at the level of papillary muscles. From M-mode tracings, anatomical parameters in diastole and systole were obtained (17). Ejection fraction (EF) was derived from LV cross-sectional area in 2D short axis view (17): $EF = [(LVDA - LVSA)/LVDA] \times 100$, where LVDA and LVSA correspond to LV areas in diastole and in systole. Mice were anesthetized with chloral hydrate (400 mg/kg body weight, i.p.), and a microtip pressure transducer (SPR-671; Millar Instruments, Houston) connected to a chart recorder was advanced into the LV for the evaluation of pressures and + and − dP/dt in the closed-chest preparation (10, 15, 16).

Cardiac Anatomy and Infarct Size. After hemodynamic measurements, the abdominal aorta was cannulated, the heart was arrested in diastole with $CdCl_2$, and the myocardium was perfused with 10% (vol/vol) formalin. The LV chamber was filled with fixative at a pressure equal to the *in vivo* measured

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: BMC, bone marrow cells; SCF, stem cell factor; G-CSF, granulocyte-colony stimulating factor; LV, left ventricle; EC, endothelial cells; SMC, smooth muscle cells; EF, ejection fraction; SO, sham-operated; LVFW, LV free wall; M, myocyte; MI, myocardial infarction.

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www.pnas.org/cgi/doi/10.1073/pnas.181177898

end-diastolic pressure (15, 16). The LV intracavitary axis was measured, and three transverse slices from the base, mid-region, and apex were embedded in paraffin. The mid-section was used to measure LV thickness, chamber diameter, and volume (15, 16). Infarct size was determined by the number of M lost from the left ventricular free wall (LVFW; refs. 18 and 19).

Newly Formed M. The volume of regenerating myocardium was determined by measuring in each of three sections the area occupied by the restored tissue and section thickness. The product of these two variables yielded the volume of tissue repair in each section. Values in the three sections were added, and the total volume of formed myocardium was obtained. Additionally, the volume of 400 M was measured in each heart. Sections were stained with desmin and laminin Abs and propidium iodide (PI). Only longitudinally oriented cells with centrally located nuclei were included. The length and diameter across the nucleus were collected in each M to compute cell volume, assuming a cylindrical shape (18, 19). M were divided in classes, and the number of M in each class was calculated from the quotient of total M class volume and average cell volume (20, 21). The number of arteriole and capillary profiles per unit area of myocardium was measured as described (18, 19).

BrdUrd and Ki67. Sections were incubated with BrdUrd or Ki67 Ab. M were recognized with a mouse monoclonal anti-cardiac myosin, endothelial cells (EC) were recognized with rabbit polyclonal anti-factor VIII, and smooth muscle cells (SMC) were recognized with a mouse monoclonal anti-α-smooth muscle actin. The fractions of M, EC, and SMC nuclei labeled by BrdUrd and Ki67 were obtained by confocal microscopy (10). Nuclei sampled in 11 cytokine-treated mice for BrdUrd were M = 3,541; EC = 2,604; SMC = 1,824; and for Ki67 were M = 3,096; EC = 2,465; SMC = 1,404.

Cell Differentiation. Cytoplasmic and nuclear markers were used; M nuclei, rabbit polyclonal Csx/Nkx2.5, MEF2, and GATA4 Abs (10, 22, 23); cytoplasm, mouse monoclonal nestin (24), rabbit polyclonal desmin (25), cardiac myosin, mouse monoclonal α-sarcomeric actin, and rabbit polyclonal connexin 43 Abs (10); EC cytoplasm, mouse monoclonal flk-1, vascular endothelial (VE)-cadherin, and factor VIII Abs (10, 26, 27); and SMC cytoplasm, flk-1 and α-smooth muscle actin Abs (10, 28). Scar was detected by a mixture of collagen type I and type III Abs.

Statistics. Results are mean ± SD. Significance was determined by the Student's *t* test and Bonferroni method (16). Mortality was computed with a log-rank test. $P < 0.05$ was significant.

Results

BMC Mobilization by Cytokines Reduces Mortality and Induces Myocardial Repair After Infarction. Given the ability of bone marrow Lin$^-$ c-*kit*^{POS} cells to transdifferentiate into the cardiogenic lineage (10), we used a protocol to maximize their number in the peripheral circulation to increase the probability of their homing to the region of dead myocardium. In normal animals, the frequency of Lin$^-$ c-*kit*^{POS} cells in the blood is only a small fraction of similar cells present in the bone marrow (13, 14). We have documented previously that the cytokine treatment used here promotes a marked increase of Lin$^-$ c-*kit*^{POS} cells in the bone marrow and a redistribution of these cells from the bone marrow to the peripheral blood. This protocol leads to a 250-fold increase in Lin$^-$ c-*kit*^{POS} cells in the circulation (13, 14).

In the current study, BMC mobilization by SCF and G-CSF resulted in a dramatic increase in survival of infarcted mice; with cytokine treatment, 73% of mice (11 of 15) survived 27 days, whereas mortality was very high in untreated infarcted mice (Fig. 1*A*). A large number of animals in this group died from 3 to 6 days after MI and only 17% (9 of 52) reached 27 days ($P < 0.001$). Mice that died within 48 h post-MI were not included in the mortality curve to minimize the influence of the surgical trauma. Infarct size was similar in the cytokine- [64 ± 11% (n = 11)] and saline- [62 ± 9% (n = 9)] injected animals as measured by the number of M lost in the LVFW at 27 days (see Fig. 5, which is published as supplemental data on the PNAS web site, www.pnas.org).

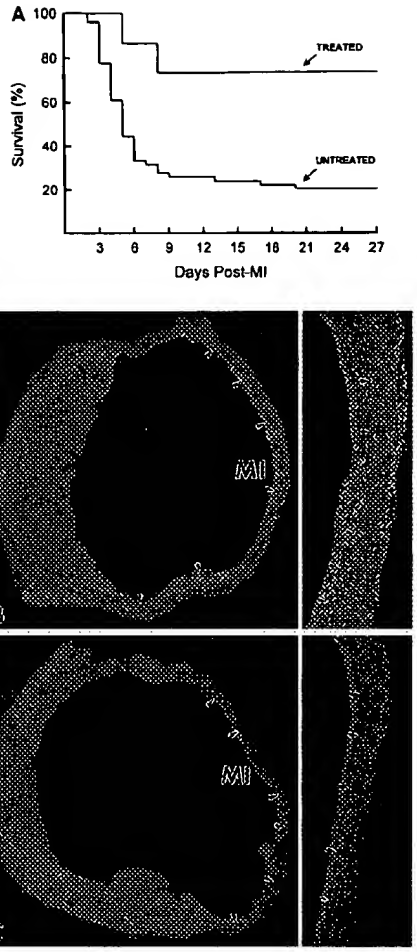

Fig. 1. Mortality and myocardial regeneration. (*A*) Cytokine-treated infarcted mice, n = 15; untreated infarcted mice, n = 52; log-rank test, $P <$ 0.0001. (*B*) Large infarct (MI) in a cytokine-treated mouse; forming myocardium (arrowheads) at higher magnification (adjacent panel). (*C*) MI in a nontreated mouse. Healing comprises the entire infarct (arrowheads). Scarring at higher magnification (adjacent panel). Red = cardiac myosin; yellow-green = propidium iodide (PI) labeling of nuclei; blue-magenta = collagen types I and III. (*B* and *C*, ×20; *Insets*, ×80.)

BMC mobilization promoted myocardial regeneration in all 11 cytokine-treated infarcted mice, killed 27 days after surgery (Fig. 1*B*). Myocardial growth within the infarct was also seen in the 4 mice that died prematurely at day 6 (n = 2) and at day 9 (n = 2). Cardiac repair was characterized by a band of newly formed myocardium occupying most of the damaged area. The developing tissue extended from the border zone to the inside of the injured region and from the endocardium to the epicardium of the LVFW. In the absence of cytokines, myocardial replacement was never observed, and healing with scar formation was apparent (Fig. 1*C*). Conversely, only small areas of collagen accumulation were detected in treated mice.

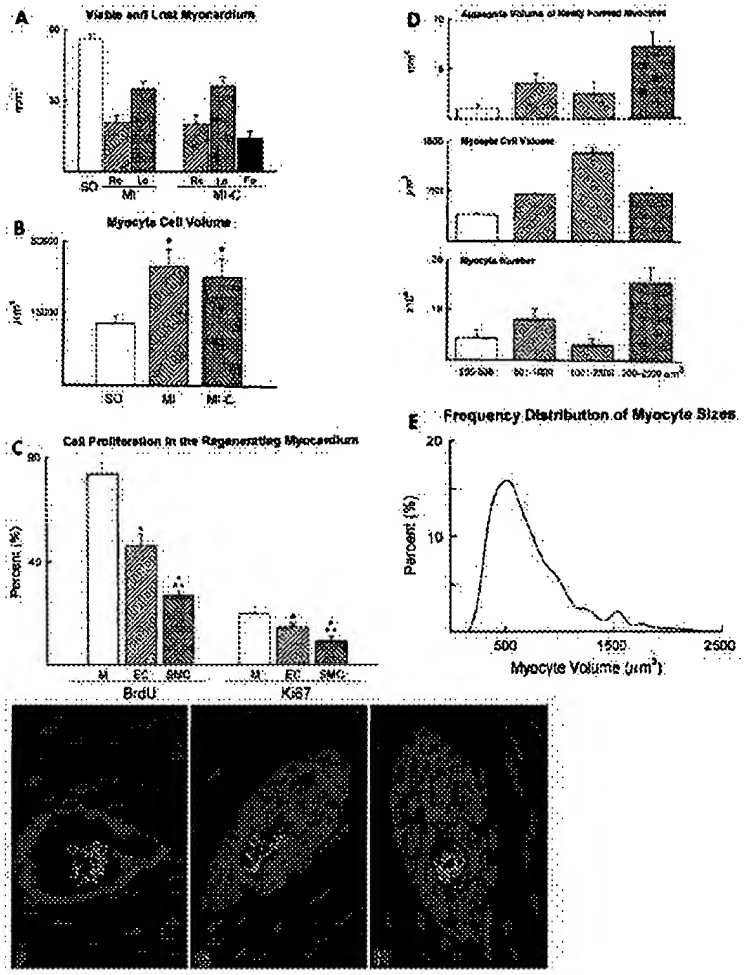

Fig. 2. Myocardial regeneration. (*A*) Remaining viable (Re), lost (Lo), and newly formed (Fo) myocardium in LVFW at 27 days in MI and MI-C; SO, myocardium without infarct. (*B*) Cellular hypertrophy in spared myocardium. (*C*) M, EC, and SMC labeled by BrdUrd and Ki67; $n = 11$. * and **, $P < 0.05$ vs. M and EC. (*D* and *E*) Volume, number ($n = 11$), and class distribution (bucket size, 100 μm^3; $n = 4,400$) of M within the formed myocardium. (*F–H*) Arterioles with TER-119-labeled erythrocyte membrane (green fluorescence); blue fluorescence = propidium iodide (PI) staining of nuclei; red fluorescence = α-smooth muscle actin in SMC. *F*, ×800; *G* and *H*, ×1,200.

BMC Mobilization Partially Restored Myocardial Mass. To quantify the contribution of the developing band to the ventricular mass, we first determined the volume of the LVFW (weight divided by 1.06 g/ml) in each group of mice. These data were 56 ± 2 mm^3 in SO, 62 ± 4 mm^3 (viable FW = 41 ± 3; infarcted FW = 21 ± 4) in infarcted nontreated animals, and 56 ± 9 mm^3 (viable FW = 37 ± 8; infarcted FW = 19 ± 5) in infarcted cytokine-treated mice. These values were compared with the expected values of spared and lost myocardium at 27 days, given the size of the infarct in the nontreated and cytokine-treated animals. From the volume of the LVFW (56 mm^3) in SO and infarct size in nontreated (62%) and treated (64%) mice, it was possible to calculate the volume of myocardium destined to remain (nontreated = 21 mm^3; treated = 20 mm^3) and destined to be lost (nontreated = 35 mm^3; treated = 36 mm^3) 27 days after coronary occlusion (Fig. 2*A*). The volume of newly formed myocardium was detected exclusively in cytokine-treated mice and found to be 14 mm^3 (Fig. 2*A*). Thus, the repair band reduced infarct size from 64% (36 mm^3/56 mm^3 = 64%) to 39% [(36 mm^3 − 14 mm^3)/56 mm^3 = 39%]. Because the spared portion of the LVFW at 27 days was 41 and 37 mm^3 in nontreated and treated mice (see above), the remaining myocardium, shown in Fig. 2*A*, underwent 95% ($P < 0.001$) and 85% ($P < 0.001$) hypertrophy, respectively. Consistently, M cell volume increased 94% and 77% (Fig. 2*B*).

Myocardial Regeneration Is Characterized by Dividing Myocytes and Forming Vascular Structures. Ki67 and BrdUrd were used to evaluate the growth stage of the cells in the regenerating band (Fig. 6 *A–D*, which is published as supplemental data on the PNAS web site). BrdUrd was injected daily between days 14–26 to measure the cumulative extent of cell proliferation while Ki67 was assayed to determine the number of cycling cells at the time of death. Ki67 identifies cells in G_1, S, G_2, prophase, and metaphase, decreasing in anaphase and telophase (10). The percentages of BrdUrd- and Ki67-positive M were 1.6- and 1.4-fold higher than EC, and 2.8- and 2.2-fold higher than SMC, respectively (Fig. 2*C*). The forming myocardium occupied 76 ± 11% of the infarct; M constituted 61 ± 12%, new vessels 12 ± 5%, and other components 3 ± 2%. The band contained 15 × 10^6 regenerating M that were in an active growing phase and had a wide size distribution (Fig. 2 *D* and *E*). EC and SMC growth resulted in the formation of 15 ± 5 arterioles and 348 ± 82 capillaries per mm^2 of new myocardium. Thick wall arterioles with several layers of SMC and luminal diameters of 10–30 μm represented vessels in early differentiation. At times, incomplete perfusion of the coronary branches within the repairing myocardium during the fixation procedure led to arterioles and capillaries containing erythrocytes (Fig. 2 *F–H*). This observation provided evidence that the new vessels were functionally competent and connected with the coronary circulation. Therefore, tissue repair reduced infarct size and M growth exceeded angiogenesis; muscle mass replacement was the prevailing feature of the infarcted heart.

Five cytoplasmic proteins were identified to establish the state

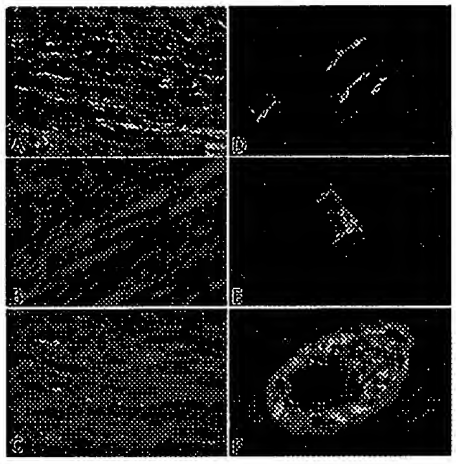

Fig. 3. Markers of differentiating cardiac cells. (*A–F*) Labeling of M by nestin (*A*, yellow), desmin (*B*, red), and connexin 43 (*C*, green); red fluorescence = cardiac myosin (*A* and *C*). (*D* and *E*) Yellow-green fluorescence reflects labeling of EC by flk-1 (arrows, *D*) and VE-cadherin (arrows, *E*); red fluorescence = factor VIII in EC (*D* and *E*). (*F*) Green fluorescence labeling of SMC cytoplasms by flk-1; endothelial lining is also labeled by flk-1; red fluorescence = α-smooth muscle actin; blue fluorescence = propidium iodide (PI) labeling of nuclei. (*A* and *E*, ×1,200; *B* and *F* ×800; *C*, ×1,400; *D*, ×1,800.)

of differentiation of M (10, 24, 25): nestin, desmin, α-sarcomeric actin, cardiac myosin, and connexin 43. Nestin was recognized in individual cells scattered across the forming band (Fig. 3*A*). With this exception, all other M expressed desmin (Fig. 3*B*), α-sarcomeric actin, cardiac myosin, and connexin 43 (Fig. 3*C*). Three transcription factors implicated in the activation of the promoter of several cardiac muscle structural genes were examined (10, 22, 23): Csx/Nkx2.5, GATA-4, and MEF2 (Fig. 7 *A–C*, which is published as supplemental data on the PNAS web site). Single cells positive for flk-1 and VE-cadherin (26, 27), two EC markers, were present in the repairing tissue (Fig. 3 *D* and *E*); flk-1 was detected in SMC isolated or within the arteriolar wall (Fig. 3*F*). This tyrosine kinase receptor promotes migration of SMC during angiogenesis (28). Therefore, repair of the infarcted heart involved growth and differentiation of all cardiac cell populations, resulting in *de novo* myocardium.

Myocardial Repair Improved Anatomical Remodeling and Ventricular Function. Myocardial regeneration attenuated cavitary dilation and mural thinning during the evolution of the infarcted heart *in vivo*. Echocardiographically, LV end-systolic (LVESD) and end-diastolic diameters (LVEDD) increased more in nontreated than in cytokine-treated mice at 9, 16, and 26 days after infarction (Fig. 8 *A* and *B*, which is published as supplemental data on the PNAS web site). Infarction prevented the evaluation of anterior wall systolic thickness (AWST) and anterior wall diastolic thickness (AWDT) . When measurable, posterior wall thickness in systole (PWST) and diastole (PWDT) was greater in treated mice (Fig. 8 *C* and *D*). Anatomically, the wall bordering and remote from infarction was 26% and 22% thicker in cytokine-injected mice (Fig. 8 *E*). BMC-induced repair resulted in a 42% higher wall thickness to chamber radius ratio (Fig. 4*A*). Additionally, tissue regeneration decreased the expansion in cavitary diameter (−14%), longitudinal axis (−5%; Fig. 8 *F* and *G*), and chamber volume (−26%; Fig. 4*B*). Importantly, ventricular mass to chamber volume ratio was 36% higher in treated animals (Fig. 4*C*). Therefore, BMC mobilization that led to proliferation and differentiation of a new population of M and vascular structures attenuated the anatomical variables which define cardiac decompensation.

Measurements of EF during the evolution of infarction and hemodynamics at the time of death showed that repair improved ventricular performance. EF was 48, 62, and 114% higher in treated than in nontreated mice at 9, 16, and 26 days after coronary occlusion, respectively (Fig. 4*D*). In mice exposed to cytokines, contractile function developed with time in the infarcted region of the wall (Fig. 4 *E–M*; Fig. 8 *H–P*). Conversely, LV end-diastolic pressure (LVEDP) increased 76% more in nontreated mice. The changes in LV systolic pressure (not shown), developed pressure (LVDP), and + and − dP/dt were also more severe in the absence of cytokine treatment (Fig. 9 *A–D*, which is published as supplemental data on the PNAS web site). Additionally, the increase in diastolic stress in the zone bordering and remote from infarction was 69–73% lower in cytokine-treated mice (Fig. 4*N*). Therefore, cytokine-mediated infarct repair restored a noticeable level of contraction in the regenerating myocardium, decreasing diastolic wall stress and increasing ventricular performance.

Discussion

On the basis of the results presented above, we conclude that cytokine administration, with the consequent mobilization of BMC into the circulation and, presumably, their translocation to the infarcted portion of the heart, led to a significant magnitude of myocardial repair. Tissue regeneration comprised parenchymal cells and vascular structures. This anatomical restoration was accompanied by a dramatic reduction in post-MI mortality and a remarkable recovery in ventricular performance. Such a high degree of anatomical and functional improvement was accomplished in 100% of the treated animals by using a noninvasive procedure. The importance of these observations for the potential treatment of ischemic heart disease in humans is apparent.

The ability of exogenous BMC to home to the damaged area of the myocardium and differentiate into cells of the cardiogenic lineage, including coronary arterioles and capillaries, was shown previously by local transplantation of Lin^- c-kit^{POS} cells into the border zone of an acute infarct (10). However, thoracic surgery and injection of foreign cells were required. Because of the complexity of the protocol, the rate of success with this invasive approach was only 40%. Additionally, this procedure required the availability of syngeneic donors as the source of the transplanted cells. In contrast, the methodology described here succeeded in all cases, eliminated the mortality and morbidity of thoracic surgery and, most importantly, obviated the use of foreign cells with the risk of transmission of infectious agents and the activation of an immunological reaction. Obviously, the generation of true myocardium and coronary vessels is superior to the use of skeletal myoblasts as a replacement for dead cardiac tissue (4, 5, 29). Although skeletal myoblasts survive and differentiate into skeletal muscle when injected into the myocardium, they never become electrically coupled with the rest of the heart because they do not express connexin 43 (4, 5, 29). Moreover, the diastolic properties of skeletal muscle cells are different from those of cardiomyocytes. Similarly, the formation of vessels only severely limits the possibility of complete functional repair after a segmental loss of ventricular mass (30).

Connexin 43 was clearly detectable in the newly formed M derived from BMC at 9 days after implantation (10) and it acquired a more mature pattern of distribution at 27 days. Consistent with the identification of contractile activity in the repairing myocardium, the expression of connexin 43 suggests that operative gap junctions were developed between M. To our knowledge, with the exception of this and our previous report using BMC, none of the published attempts to repair cardiac tissue post-MI have resulted in the production of functional, healthy myocardium. Although not investigated here, this con-

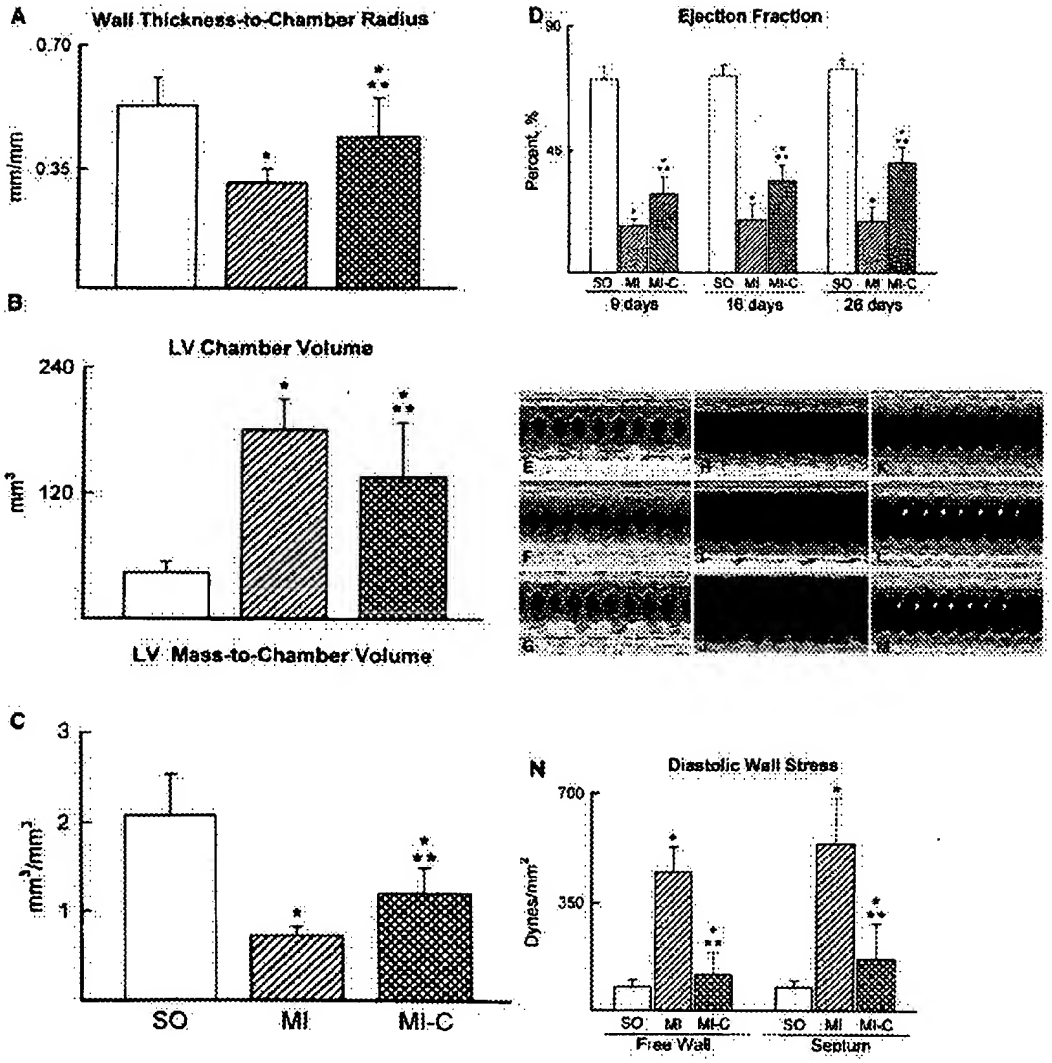

Fig. 4. MI, cardiac anatomy, and function. (*A–C*) LV dimensions at time of death, 27 days after surgery; SO (n = 9), nontreated infarcted (MI, n = 9), and cytokine-treated infarcted (MI-C, n = 10). (*D*) EF by echocardiography (SO, n = 9; MI, n = 9; and MI-C, n = 9). (*E–M*) M-mode echocardiograms of SO (*E–G*), MI (*H–J*), and MI-C (*K–M*); newly formed contracting myocardium (arrows). Detailed echocardiograms are shown in Fig. 8. (*N*) Wall stress, SO (n = 9); MI (n = 8); and MI-C (n = 9). Results are mean ± SD. * and **, $P < 0.05$ vs. SO and MI, respectively.

tention implies that extracellular matrix supporting parenchymal cells and coronary vessels had to be formed. Additionally, scar formation was minimal in the treated animals but this does not exclude the notion that groups of myofibroblasts were present at the edges of the regenerating tissue.

The long-term unfavorable outcome of the infarcted heart is directly related to the initial infarct size that determines the degree of impaired pump function and the magnitude of dilation and wall thinning (31, 32). The changes in cardiac anatomy acutely after infarction, in combination with elevated filling pressure and decreased systolic pressure, induce large increases in diastolic stress and modest increases in systolic stress (18, 31). These structural-functional modifications promote chronic remodeling and the evolution of the myopathy to terminal failure (19, 31). Formation of new myocardium within the infarct attenuated the anatomical alterations, led to chronic increases in EF, and reduced the abnormalities in cavitary pressure, contractility, and loading. Longer intervals after homing of primitive BMC may result in complete repair of the infarcted heart.

Despite the success of the protocol used here, there are questions that we are currently addressing. Administration of SCF and G-CSF mobilizes pluripotent Lin$^-$ c-*kit*^{POS} cells from the bone marrow to the peripheral blood (13). The number of circulating Lin$^-$ c-*kit*^{POS} cells increased 250-fold. Donor BMC injected intravenously home to injured organs including liver (8) and skeletal muscle (33). Because of previous results with Lin$^-$ c-*kit*^{POS} cells (10), we propose that Lin$^-$ c-*kit*^{POS} cells are responsible for cardiac repair. Our results do not provide unambiguous information about the origin of the cells reconstituting the myocardium. It could be argued that cytokine treatment mobilized bone marrow stem cells and resident cardiac stem cells, which together participated in tissue regeneration. We have begun gene-marking studies in an effort to document

the plasticity of adult Lin$^-$ c-*kit*^{POS} bone marrow cells in myocardial repair. This issue could also be addressed by treatment of irradiated animals with cytokines or by transfusing infarcted animals with BMC from syngeneic sex-mismatched donors.

The efficacy of cytokine treatment starting 5 days before MI followed by 3 more days postcoronary occlusion raises the question of the most effective therapeutic window. Additionally, it is not clear whether tissue repair is a result of the homing of BMC to the lesion or whether BMC, once mobilized, nest randomly throughout the organism and only those in the damaged myocardium rapidly proliferate and transdifferentiate. The former possibility is more attractive and supported by the rapid induction of SCF in a number of tissues, including the myocardium (34), in response to injury (34–37). SCF could be responsible for migration, accumulation, and multiplication of primitive BMC in the infarcted zone where they acquire the heart muscle phenotype reaching functional competence.

In conclusion, BMC injected or mobilized to the damaged myocardium behave as cardiac stem cells, giving rise to M, endothelial cells, and smooth muscle cells. Such behavior is no longer surprising given the remarkable plasticity of adult bone marrow stem cells (8–12, 30). New evidence offers clues as to some of the biochemical pathways implicated in this transdifferentiation. The interplay between the signal transduction pathways of bone morphogenetic proteins and the *Wnt* family of genes is responsible for the expression of lineage-determining genes that condition whether a mesodermal precursor cell becomes a blood cell or a cardiac cell (38, 39). During development, the differentiation into a M or a hemopoietic cell is an opposing and mutually exclusive choice. This choice, established by the nature of the cell, seems to be influenced by cues from the environment where the cell resides. Such environmental cues have proven to be difficult to elucidate in the maturing embryo. Surprisingly, as shown here and previously (10, 13), adult BMC remain open to both developmental pathways and readily reprogram themselves in response to the habitat. The absence of hematopoietic islands in the regenerating myocardium as a result of BMC localization is further testimony of the responsiveness of these cells to environmental factors. Thus, this system offers a favorable experimental setting to uncover the biology of this intriguing and potentially clinically useful transdifferentiation.

We believe that the approach presented here might bring myocardial regeneration closer to clinical reality and might also offer the opportunity to uncover the molecular mechanisms involved.

We thank Dr. Seigo Izumo (Beth Israel Deaconess Medical Center, Boston) for providing us with the Csx/Nkx2.5 Ab. The mAb Rat-401 (anti-nestin) developed by S. Hockfield was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development, and maintained by the University of Iowa, Iowa City. This work was supported by National Institutes of Health Grants HL-38132, HL-39902, HL-43023, AG-15756, AG-17042, HL-66923, and HL-65577.

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